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L4	L3 and label$	1	L4
L3	l2 and probe$1	1	L3
L2	multiple mutation$1 near5 target near5 nucleic acid	1	L2
L1	multiple mutations near5 single near5 target nucleic acid$1	0	L1

END OF SEARCH HISTORY

End of Result Set

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L4: Entry 1 of 1 File: USPT Dec 30, 1997

DOCUMENT-IDENTIFIER: US 5702931 A
TITLE: Mutagenesis methods and compositions

Brief Summary Text (7):
The most general method for mutant screening is by hybridizing DNA from cells transformed with the double-stranded mutagenesis product with a 5'-labeled mutagenic oligonucleotide (R. Wallace et al., Science 209:1396 (1980), incorporated herein by reference). Under nonstringent conditions (e.g., room temperature wash) the probe hybridizes both to the mutant DNA to which it is perfectly matched and also to wild-type DNA to which it is mismatched. By increasing the stringency of washing (e.g., by elevating the temperature) the mutagenic oligonucleotide can be selectively dissociated from wild-type DNA, leaving it bound to mutant DNA. DNA is then prepared and sequenced to verify the mutation. If one employs oligonucleotide-directed mutagenesis methods which result in a high proportion of transformed cells bearing mutant DNA, one can often use sequencing itself as a mutant screen.

Brief Summary Text (11):
The present invention provides a significant improvement over previously described oligonucleotide-directed mutagenesis efforts, addressing these and other needs, providing methods and kits for rapidly and easily site-specifically modifying a target nucleic acid, methods especially well suited to introducing multiple mutations at one or several positions on the target nucleic acid.

Detailed Description Text (33):
It will be readily appreciated that one may employ multiple mutagenic oligonucleotides to mutate several sites on a polypeptide encoded by a target nucleic acid. One may, in this circumstance, obtain a majority of the mutations desired, but fail to obtain all the desired mutations in a single mutagenesis round. One can readily obtain the remaining mutations by sequential mutations of the same template by employing, for instance, the dual marker system described above, without subcloning the target nucleic acid to be mutated. The time and effort required to generate multiple mutations in a target nucleic acid is dramatically simplified by the use of the methods of the present invention.

Generate Collection

L13: Entry 15 of 16 File: USPT Jul 4, 2000

DOCUMENT-IDENTIFIER: US 6083763 A
TITLE: Multiplexed molecular analysis apparatus and method

Brief Summary Text (13):
Blotting techniques, such as those used in Southern and Northern analyses, have been used extensively as the primary method of detection for clinically relevant nucleic acids. The samples are prepared quickly to protect them from endogenous nucleases and then subjected to a restriction enzyme digest or polymerase chain reaction (PCR) analysis to obtain nucleic acid fragments suitable for the assay. Separation by size is carried out using gel electrophoresis. The denatured fragments are then made available for hybridization to labeled probes by blotting onto a membrane that binds the target nucleic acid. To identify multiple fragments, probes are applied sequentially with appropriate washing and hybridization steps. This can lead to a loss of signal and an increase in background due to non-specific binding. While blotting techniques are sensitive and inexpensive, they are labor intensive and dependent on the skill of the technician. They also do not allow for a high degree of multiplexing due to the problems associated with sequential applications of different probes.

Brief Summary Text (25):
3) Problems with labeling systems are often problematic in sandwich assays. Sandwich assays, consisting of labeled probes complementary to secondary sites on the bound target molecule, are commonly used in hybridization experiments. These sites are subject to the above mentioned binding domain problems. Enzymatic chemiluminescent systems are subject to inhibitors of the enzyme or substrate and endogenous peroxidases can cause false positives by oxidizing the chemiluminescent substrate.

Brief Summary Text (42):
Multiple DNA/RNA probe arrays can be fabricated in the bottom of 96 well microtiter plates which offer the potential of performing 1,536 (96.times.16) to 21,600 (96.times.225) hybridization tests per microtiter plate. Each well will contain a probe array of N elements dispensed onto plastic or glass and bonded to the microtiter plate. Moreover, by coupling the microtiter trays to a direct (lensless) CCD proximal/imager, all 1,536 to 21,600 hybridization tests can be quantitatively accessed within seconds at room temperature. Such proximal CCD detection approach enables unprecedented speed and resolution due to the inherently high collection efficiency and parallel imaging operation. The upper limit to the hybridization tests per microtiter plate exceeds 100,000 based on a 100 .mu.m center-to-center spacing of biosites.

Brief Summary Text (46):
The multiplexed assay can be designed in a standard 96 well microtiter plate format for room temperature operation to accommodate conventional robotic systems utilized for sample delivery and preparation. Also, the proximal CCD-based imager with a graphical user interface will enable the automation of the parallel acquisition of the numerous hybridization test results. The CCD imaging system software provides automated filtering, thresholding, labeling, statistical analysis and quantitative graphical display of each probe/target binding area within seconds.

Brief Summary Text (48):
The proximal CCD detector/imager utilized in a particular embodiment of the multiplexed molecular analysis system accommodates numerous molecule labeling strategies including fluorescence, chemiluminescence and radioisotopes. Consequently, a single instrument can be employed for a variety of reporter groups used separately or together in a multiplexed manner for maximal information extraction.

Detailed Description Text (10):

The term "probe arrays" refers to the array of N different biosites deposited on a reaction substrate which serve to interrogate mixtures of target molecules or multiple sites on a single target molecule administered to the surface of the array.

Detailed Description Text (16):
By "multiplexed diagnostic assay" is meant a method for performing in parallel a large set or number of diagnostic assays. Thus a set of parallel reactions can be handled with the same effort as a single sample in previously described methods. Hence, a greater number of assays can be handled within a fixed period of time. The parallel set of reactions or multiplexed assay must be deciphered at the end of the process. This is done by labeling or tagging the biosite, as defined herein.

Detailed Description Text (26):
By "methods of detecting (or detection) the association/hybridization" is meant to include, without limitation, fluorescent labeling, radioisotope labeling, chemiluminescence labeling, bioluminescence labeling, colorimetric labeling. Labeling can be achieved by one of the many different methods known to those with skill in this art.

Detailed Description Text (27):
The term "luminescence" refers to, without limitation, electrical (electro), chemical, fluorescence, phosphorescence, bioluminescence, and the like. However, for this invention, electrochemiluminescence or electrical chemiluminescence (ECL) labeling is included as another method of detection which does not require a wash step to remove excess target molecules from the solution, and is highly sensitive. For the electrochemiluminescence or electrical chemiluminescence method of detection, once hybridization/association has occurred and a voltage has been applied, only the labeled target molecules associated with the biosite will emit light and be detected. The residual excess label in the solution not associated with the biosite will therefore not emit light.

Detailed Description Text (31):
A. Preparing the sample for subsequent association to a probe array within the reaction chamber. This includes all front-end processes such as purification, isolation, denaturation and labeling required to extract the target molecules from the sample.

Detailed Description Text (38):
STEP 3. Molecular Labeling Strategies. Molecular labeling strategies relates to versatile labeling of the target molecules (fluorescence, chemiluminescence, etc.) consistent with proximal large area detection/imaging.

Detailed Description Text (60):
An important feature of the capillary bundle printer is the manner in which it interfaces to the printing solution storage vessel. The capillary bundles have a printing (distal) end and a storage vessel end. The printing solution is held in a sealed container that positions every capillary in the printing bundle via a manifold so that each capillary dips into a specific well (supply chamber) of a microtiter plate, one capillary per well. Current multi-well microtiter plates are available with 96, 384, or 1536 wells, and can contain up to 96, 384, or 1536 individual probe solutions, respectively. For microarrays containing more probe elements, multiple printing solution reservoirs or storage vessels can be interfaced to a single print head, as illustrated in FIG. 4a. This design concept eliminates the geometry problems associated with load and dispense systems. The flexible fused silica capillaries can be gathered together with the array templates or sleeves to create a print head with capillaries spaced as close as 200 .mu.m center to center.

Detailed Description Text (66):
The prime and continuous print with multiple capillaries prevents contamination of the probe solution that can occur with load and dispense systems, which must contact the surface and then return to the probe solution to draw more fluid. The continuous printing of the capillary bundle printer is extremely efficient and proves to be an enabling technique for applications that require the use of small volumes of probe solution. The small outer and inner diameters of the printing capillaries allow for printing as many as 10,000 spots per .mu.L from a total volume of less than 5 .mu.L.

Detailed Description Text (121):
A Universal Array having 16 capture probes within a single well of a 96 well microtiter plate is shown in FIG. 5c. FIG. 5c is a printed computer image showing a multi-microtiter well proximal CCD image of a 4.times.4 Universal Array. In FIG. 5c, target specific hybridization is observed in 15 out of the 16 oligo elements in the array. The results of 15 target specific hybridizations conducted simultaneously in 3 separate reaction chambers in a multiwell reaction vessel are quantitatively assessed from the digital image obtained from the proximal CCD imager. Hybrids are digoxigenin end-labeled oligonucleotide targets detected using anti-digoxigenin antibody-alkaline phosphatase conjugate and ELF.TM. fluorescence. In this assay (from Molecular Probes, Inc.) the antibody binds to the digoxigenin group, delivering alkaline phosphatase to the bound target. The alkaline phosphatase converts the non-fluorescent ELF precursor to a fluorescent product which can be detected by UV irradiation.

Detailed Description Text (122):
FIG. 5d is a printed computer image showing a single microtiter well proximal CCD image of a 4.times.4 universal array. FIG. 5d shows the target specific hybridization of 4 of the 16 oligonucleotide elements in the array at positions A2, B2, C2, and D2. Note the desirable absence of significant cross hybridization, which has been specifically minimized by imposing the maximum dissimilarly design constraints. Hybrids are digoxigenin end-labeled oligonucleotide targets detected using anti-digoxigenin alkaline phosphatase conjugate and ELF.TM. fluorescence as described above.

Detailed Description Text (124):
Small molecule Universal Arrays can be employed for rapid, high throughput drug screening. In this format, surface bound capture probes consist of small haptens or molecules arranged in separated biosites attached to a solid support. Each biosite consists of specifically-addressable, covalently immobilized small molecules such as haptens, drugs and peptides. These organic capture molecules are designed to have a high affinity association with a bispecific ligand. These ligands contains both a domain cognate to the small immobilized organic molecule (capture probe) and cognate to the analyte of interest. The domain cognate to the analyte can associate either directly to this target or to a label on the analyte.

Detailed Description Text (127):
Conversely, the format for a small molecule Universal Array can be inverted so that the macromolecular ligand becomes the capture probe. Thus, a Universal Array (Macromolecular Universal Array) may contain large macromolecules such as, without limitation, antibodies, proteins, polysaccachrides, peptides, or receptors as the immobilized capture probe. In turn, unique small molecule tags having a specific, high affinity association for the macromolecular biosites are covalently attached to various probes cognate to the analyte. These labeled probes now represent the bispecific component cognate to both the capture macromolecule and the target analyte. Some representative examples of small molecules (haptens or drugs) are listed in Table 1 below. This is only a partial list of commercially available antibodies to haptens, steroid hormones and other small molecule drugs. Examples of these bispecific, small molecule-labeled macromolecules include antibodies, receptors, peptides, oligonucleotides, dsDNA, ssDNA, RNA, polysaccharides, streptavidin, or lectins. A partial list of 48 representative compounds for which specific antibodies are available include: fluorescein; dinitrophenol; amphetamine; barbiturate; acetaminophen; acetohexamide; desipramine; lidocaine; digitoxin; chloroquinine; quinine; ritalin; phenobarbital; phenytoin; fentanyl; phencyclidine; methamphetamine; metaniphrine; digoxin; penicillin; tetrahydrocannibinol; tobramycin; nitrazepam; morphine; Texas Red; TRITC; primaquine; progesterone; bendazac; carbamazepine; estradiol; theophylline; methadone; methotrexate; aldosterone; norethisterone; salicylate; warfarin; cortisol; testosterone; nortrptyline; propanolol; estrone; androstenedione; digoxigenin; biotin; thyroxine; and triiodothyronine.

Detailed Description Text (129):
Step 3--Molecular Labeling Strategies

Detailed Description Text (130):
Molecular labeling strategies relate to versatile labeling of the target molecules

(fluorescence, chemiluminescence, etc.) consistent with proximal large area detection/imaging.

Detailed Description Text (131):
1. Introduction--Conventional Labeling

Detailed Description Text (132):
Labeling can be achieved by one of the many different methods known to those skilled in the art. In general, labeling and detection of nucleic acid hybrids may be divided into two general types: direct and indirect. Direct methods employ either covalent attachment or direct enzymatic incorporation of the signal generating moiety (e.g., isotope, fluorophore, or enzyme) to the DNA probe. Indirect labeling uses a hapten (e.g., biotin or digoxigenin) introduced into the nucleic acid probe (either chemically or enzymatically), followed by detection of the hapten with a secondary reagent such as streptavidin or antibody conjugated to a signal generating moiety (e.g., fluorophore or signal generating enzymes such as alkaline phosphatase or horseradish peroxidase).

Detailed Description Text (133):
For example, methods of detecting the association/hybridization include, without limitation, fluorescent labeling, radioisotope labeling, chemiluminescence labeling, bioluminescence labeling, colorimetric labeling and electrochemiluminescence labeling. Many known labeling techniques require a wash step to remove excess target from the hybridization/association solution, e.g., fluorescent, radioisotope, chemiluminescence, bioluminescence and colorimetric labeling. Several of these will be described below.

Detailed Description Text (134):
2. Fluorescent Labeling

Detailed Description Text (135):
Fluorescent labeling is suitable for this invention for several reasons. First, potentially hazardous substances such as radioisotopes are avoided. Furthermore, the fluorescent labeling procedures are simpler than chemiluminescent methods since the latter requires enzymatic reactions and detection in the solution state. Finally, the fluorescent labeling approach can be modified to achieve the highest signal-to-nose ratio SNR among the safest labeling techniques by utilizing secondary linker chemistries that enable the attachment of hundreds of fluorescent dye molecules per target molecule.

Detailed Description Text (136):
The particular fluorescent dyes to be considered include commercially available agents such as ethidium bromide, as well as the novel dyes proposed in the affiliated chemistry component. These labeling agents have intense absorption bands in the near UV (300-350 nm) range while their principle emission band is in the visible (500-650 nm) range of the spectrum. Hence, these fluorescent labels appear optimal for the proposed proximal CCD detection assay since the quantum efficiency of the device is several orders of magnitude lower at the excitation wavelength (337 nm) than at the fluorescent signal wavelength (545 nm). Therefore, from the perspective of detecting luminescence, the polysilicon CCD gates have the built-in capacity to filter away the contribution of incident light in the UV range, yet are very sensitive to the visible luminescence generated by the proposed fluorescent reporter groups. Such inherently large discrimination against UV excitation enables large SNRs (greater than 100) to be achieved by the CCDs.

Detailed Description Text (137):
3. Electrochemiluminescence Labeling

Detailed Description Text (138):
Electrochemiluminescence or electrical chemiluminescence (ECL) labeling, e.g., ruthenium (Ru) does not require a wash step to remove excess target from the solution and is highly sensitive. Briefly, for electrochemiluminescence as a method of detection, the internal surface of the reaction chamber is coated with a conductive material, e.g., gold, and the biosite is attached to this conductive surface (See FIG. 6). FIG. 6 is a diagram showing ECL implementation in reaction vessel with proximal

CCD imaging. Using one microtiter well (of a 96 microtiter well plate) as a reaction chamber, the biosites are deposited onto the internal circumference of the microtiter well by one of several methods as described above (ink-jet, capillary, or photolithography/capillary).

Detailed Description Text (139):
This conductive surface acts as a cathode (positive lead), and an anode (negative lead) is provided by inserting a metal cup with an electrode protruding through its center into the reaction chamber (microtiter well). The electrode is positioned such that it is inserted into the hybridization solution. The voltage applied to the anode induces an electrochemical event at the labeled molecule surface which releases energy in the form of photons (light).

Detailed Description Text (140):
The specific ECL label, e.g., Ru, is attached to the target molecule by the conventional means. The labeled target is added to the hybridization solution and once hybridization occurs between the Ru labeled target and biosite, e.g., after sufficient time has passed for hybridization to be

Detailed Description Text (141):
completed, a voltage is applied and only Ru labeled target associated (hybridized) with the biosite will emit light and be detected. In order for the Ru labeled target to be detected, it must be in proximity to the cathode. The residual excess Ru labeled target not associated with the biosite will therefore not emit light.

Detailed Description Text (143):
4. Lanthanide Chelate Labeling

Detailed Description Text (158):
Moreover by placing the imaging array in proximity to the sample as illustrated in FIG. 1, the collection efficiency is improved by a factor of at least ten (100 over any lens-based technique such as found in conventional CCD cameras). Thus, the sample (emitter or absorber) is in near contact with the detector (imaging array), thereby eliminating conventional imaging optics such as lenses and mirrors. This apparatus can be used for detecting and quantitatively imaging radioisotope, fluorescent, and chemiluminescent labeled molecules, since a lensless CCD array apparatus is highly sensitive to both photons and x-ray particles. Hence a single imaging instrument can be used in conjunction with numerous molecular labeling techniques, ranging from radioisotopes to fluorescent dyes.

Detailed Description Text (168):
A preferred embodiment of the detection/imaging sensor array of the invention consists of a plurality of CCD arrays CCD1 . . . CCDN assembled in a large format module as illustrated in FIGS. 10A-10C. FIG. 10A depicts a CCD array with multiple pixels being exposed to a labeled biological sample 32 which causes the collection of electrons 34 beneath the respective pixel gate 16. Individual CCD arrays are closely aligned and interconnected in particular geometries to form a relatively large (greater than 1 cm.sup.2) format imaging sensors of the linear array type as shown in FIG. 10B or the two dimensional row and column type as shown in FIG. 10C.

Detailed Description Text (171):
Preferably, in a labeled molecule embodiment, a filter shown in dotted lines 17, which may be formed of an aluminum or tungsten metal grating, or dielectric multilayer interference filter, or absorption filter, is formed in the dielectric layer 18 between the surface and the metal electrode 16. The filter is adapted to block the excitation radiation and pass the secondary emission from the sample 20. In a static platform embodiment, the sensor module remains fixed with respect to the sample. Hence to achieve the relatively large imaging format, a plurality of imaging devices CCD1 . . . CCDN should be arranged in a module as illustrated in FIGS. 10B and 10C. The module can be packaged for easy installation to facilitate multiple modules, each for specific applications. Various tiling strategies have been documented and can be employed to minimize the discontinuity between devices, such as described in Burke, et al., "An Abuttable CCD Imager for Visible and X-Ray Focal Plane Arrays," IEEE Trans On Electron Devices, 38(5):1069 (May, 1991).

Detailed Description Text (172):
As illustrated in FIG. 10A, a reaction vessel 20 is placed in proximity to the CCD array sensor 10. The sample can be excited by an external energy source or can be internally labeled with radioisotopes emitting energetic particles or radiation, or photons may be emitted by the sample when labeled with fluorescent and chemiluminescent substances. Conversely, direct absorption may be used to determine their presence. In this case, the absence of illuminating radiation on the detector may constitute the presence of a particular molecule structure. Preferably the sample can be physically separated from the CCD detector by the faceplate which is transparent to the particle emission.

Detailed Description Text (174):
Silicon based CCDs are preferred as the solid state detection and imaging sensor primarily due to the high sensitivity of the devices over a wide wavelength range of from 1 to 10,000 .ANG. wavelengths. That is, silicon is very responsive to electromagnetic radiation from the visible spectrum to soft x-rays. Specifically for silicon, only 1.1 eV of energy is required to generate an electron-hole pair in the 3,000 to 11,000 .ANG. wavelength range. Thus for visible light, a single photon incident on the CCD gate 16 will result in a single electron charge packet beneath the gate, whereas for soft x-rays, a single beta particle (typically KeV to MeV range) will generate thousands to tens of thousands of electrons. Hence the silicon CCD device provides ultrasensitive detection and imaging for low energy alpha or beta emitting isotopes (.sup.3 H, .sup.14 C, .sup.35 S) as well as high energy alpha or beta emitting isotopes (.sup.32 P, .sup.125 I). Consequently, the CCD is both a visible imager (applicable to fluorescent and chemiluminescent labeled molecular samples) and a particle spectrometer (applicable to radioisotope labeled samples as well as external x-ray radiated samples). Thus, the CCD can provide simultaneous imaging and spectroscopy in the same image.

Detailed Description Text (195):
In a preferred embodiment, thin (50-300 .mu.m) vinyl substrates are amino or epoxy functionalized with silanes similar to glass substrates. Thin vinyl substrates are immersed in a 1-2% aqueous solution of polyvinyl alcohol at 65.degree. C. The adsorbed polyvinyl alcohol is then reacted with either epoxy silane or amino silane, thus functionalizing the polymeric hydroxyl groups. Such optically clear vinyl substrates have the distinct advantage of blocking a large amount of the UV excitation source incident on the proximal CCD detector, but allowing the longer wavelengths (e.g. 500-650 nm) to pass through efficiently. This allows for greater sensitivity of labeled detector molecules that emit in such wavelength region.

Detailed Description Text (202):
Universal Arrays are perfectly suited for analysis and detection of multiple point mutations within a single PCR template. Often technical constraints are encountered when attempting to analyze multiple point mutations from a single PCR amplicon reaction. Most point mutation analysis techniques such as ribonuclease protection assay, SSCP, or CLEAVASE.TM. are well suited for detecting a single point mutation per amplicon or DNA template and require lengthy gel-based separation techniques. The simultaneous, rapid detection of numerous point mutations within a single PCR amplicon without an expensive, lengthy gel separation step is well beyond the capability of these technologies. Other newer, non-gel based technologies such as TAQMAN.TM. are also poorly suited for multiplexed analysis within a single reaction vessel. FIG. 13 illustrates the concept of using Universal Arrays for point mutation analysis (genotyping) at a single loci. FIG. 13 is a diagram showing genotyping by universal point mutation scanning. For example purposes only, FIG. 13 uses a single point mutation biosite to illustrate this type of analysis but could just as easily be simultaneously carried out on 25 different loci on a single PCR template as illustrated in FIG. 14.

Detailed Description Text (203):
Briefly, as shown in FIG. 13, the PCR template is aliquoted into 4 separate tubes (one for each dNTP) containing a standard sequencing mix, with the exception that dideoxynucleotides are not included. Instead, a single alpha-thio dNTP is substituted in each of the four separate mixes. Each mix also contains a single labeled primer with a universal sequence or "handle" at the 5' end which anneals just one nucleotide away from the mutation site on the PCR template (note: multiple primers each with

unique universal sequences and complementary to different loci on the template is readily accomplished). After standard thermal cycle extension reactions are complete each tube is briefly incubated with snake venom phosphdiesterase. Only primers and templates that were not extended during the sequencing reaction are vulnerable to digestion by this 3'-specific exonuclease. Mutation primers containing a 3' thiophosphate ester linkage are highly resistant to digestion.

Detailed Description Text (204):
In this specific example, only the A reaction extended since a T was the next complementary base on the PCR template. Each digested, sequencing reaction mix in turn is then hybridized to four microtiter wells each containing identical immobilized microarrays complementary to the universal primer sequences. In this case, only the microtiter well hybridized to the A reaction mix gives a positive signal at a biosite loci complementary to the universal handle. In this fashion, up to 96 loci could be probed for point mutations on a single PCR template. Both strands in the PCR amplicon could be "scanned" in this manner simultaneously to allow more room for many primers to anneal without competition for the same hybridization loci on the template. In FIG. 13, "5-DIG" means 5' digoxigenin labeled.

Detailed Description Text (205):
For probe based diagnostics where both multiplexing within a single target molecule and low target concentrations are a problem, amplification with either PCR or LCR using the microtiter plate in a microtiter well concept conjoined to the Universal Array has distinct advantages. In a preferred embodiment, universal "handles" can be synthesized directly on the 5' end of Polymerase Chain Reaction or Ligase Chain Reaction primers and following in situ thermal cycling the amplified products can be simultaneously hybridized to 96 separate biosites. This format has other diagnostic advantages such as homogeneous detection of amplified products without having to open or expose the sample well to the ambient environment.

Detailed Description Text (208):
FIG. 15 illustrates this homogenous multiplexed approach for the Polymerase Chain Reaction (PCR) simultaneously at 3 different loci. FIG. 15 is a diagram showing homogeneous in situ microarray detection of multiplexed PCR amplicons. FIG. 15 illustrates specific multiplex hybridization detection of PCR products using microtiter-based microarrays. Briefly, in this figure three separate amplification loci are being detected simultaneously. Each locus (e.g, PCR LOCUS 1) is defined by two specially modified amplification primers that define the ends of the amplified PCR product. One primer in the pair, contains a fluorescently detectable label such as fluorescein. The other primer in the pair contains two domains, one is a unique universal sequence complementary to a capture probe arrayed at the bottom of a single microtiter well and the other domain specific for template amplification. The universal sequence is attached to the amplification primer in a 5' to 5' linkage so that when the polymerase is amplifying the region of interest it does not jump over this specialized juncture, leaving the universal sequence as a single stranded motif. If a particular template in a sample well being amplified contains both primer loci (i.e., detection and capture sites), then a PCR product will be generated that can simultaneously hybridize and be detected to a complementary member of a universal capture array by the CCD proximal detector. Since only PCR amplicons hybridized to members of the universal array at the bottom of each well are proximal to the detector, the assay requires no special separation step to detect hybridized amplicons and thus becomes homogenous in nature.

Detailed Description Text (209):
Similarly, FIG. 16 illustrates this multiplexed concept with Gap-Ligase Chain Reaction (G-LCR). FIG. 16 is a diagram showing homogeneous in situ microarray detection of multiplexed gap-ligase chain reaction products. The ability to detect hybridization events homogeneously is provided by the fact that only molecules proximally associated with specific biosites can be imaged by the detector. FIG. 16 illustrates specific multiplex hybridization detection of Gap-Ligase Chain Reaction products using microtiter-based microassays. Similarly, as described previously for PCR products (see FIG. 15), this figure illustrates the assay at three separate ligation-dependent amplification loci simultaneously. Each locus (e.g., LOCUS 1) is defined by two specially modified primers that define the ends of the gap ligase chain reaction product. One primer in the pair, contains a fluorescently detectable label such as

fluorescein. The other primer in the pair contains two domains, one is a unique universal sequence complementary to a capture probe arrayed at the bottom of a single microtiter well and the other domain is specific for a region on the template being detected. The universal sequence attached to this primer serves as a sequence specific single stranded handle. When the template is present in the sample then sequence directed ligation will join both the label and the universal handle into a single product. After many cycles this amplified ligated product can be simultaneously hybridized and detected to its complementary member on a universal capture array immobilized to the bottom of a microtiter well and imaged by the CCD proximal detector. Since only ligated products hybridized to members of the universal array at the bottom of each well are proximal to the detector, the assay requires no special separation step to detect hybridized amplicons and thus becomes homogenous in nature.

Detailed Description Text (215):
The bispecific ligand is specific to both the immobilized hapten or drug on the substrate surface (biosite) and the analyte being screened. Examples of Universal Array screening are diagramed in FIG. 17. FIG. 17 is a diagram showing small molecule universal array (drug screening/discovery). FIG. 17 illustrates the basic small molecule Universal Array concept using four different immobilized haptens in a single well. Various bispecific molecules are diagramed for illustration purposes. FIG. 17 illustrates four separate and distinct haptens immobilized at the bottom of each of 96 wells of a microtiter plate. Each locus or biosite in the array is defined by four unique immobilized haptens illustrated in this example by fluorescein, digoxigenin, 2,4 dinitrophenol, and TRITC. Bispecific molecules uniquely specific for both the immobilized hapten and another labeled analyte in the sample are added to each well. In this fashion, different multiple analytes can be simultaneously detected and their presence indicated by signals at specific hapten biosites. In this example, 96 individual samples can be assayed for four different analytes simultaneously. As shown, the fluorescein biosite detects a labeled receptor (protein) analyte, both the 2,4 dinitrophenol and digoxigenin haptens allow for the simultaneous detection or presence of two additional types of protein receptors in the sample. Finally, the TRITC hapten allows for detection and presence of a specific enzyme substrate via an intervening enzyme conjugate. Once again, the proximal mode of detection allows for homogenous imaging of only those binding events at the surface of the array. The advantages of such a multiplexed immunological approach is the exquisite specificity and variety of small molecules that comprise such a Universal Array using non-DNA based recognition of biosites.

Detailed Description Text (222):
Conversely, intact cells are analyzed utilizing the multiplexed format of this invention. Specifically, most "cell enrichment" protocols involve either double label flow cytometry, or physical separation of cells via affinity chromatography of some kind. Both require access to an antibody which is specific to the cell type of interest.

Detailed Description Text (224):
The procedure is to add a complex cellular mixture, e.g., a biological sample (for example, blood or sputum), to such an antibody matrix, then with some local mixing, allowing the cells to bind to the surface. If cells bind to such a matrix with good affinity and selectivity, they are then fixed to the matrix, permeabilized, and subjected to labeled probe hybridization (or PCR) in a fashion which is nearly identical to the methods which are currently used to analyze DNA or RNA in cells for microscopy or flow cytometry.

Detailed Description Text (229):
Basically, the procedure is initiated by preparing the microbial rRNA sample for hybridization to the biosite array within the reaction chamber. Following specific binding of the fluorescently labeled microbial RNA to the probe array, a two dimensional image results that uniquely characterizes the sample. The analyzer output is the microbial spectrum, consisting of the amount and type of microorganisms present in the sample.

Detailed Description Text (232):
2) Simultaneous microbial monitoring can be achieved due to the high density arrays that support hundreds of immobilized probes per cm.sup.2 to facilitate multiple

microorganism detection and identification in a high throughput manner.

End of Result Set

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L9: Entry 1 of 1 File: DWPI Jul 18, 2000

DERWENT-ACC-NO: 2000-523756
DERWENT-WEEK: 200047

TITLE: Determining number of repeat base sequences in a target oligonucleotide for diagnosing a disease, by detecting multiple mutation, utilizing continuous or contiguous stacking hybridization

Basic Abstract Text (5):
(c) contacting a labeled oligonucleotide extender molecule (IIIb) to (II), where (IIIb) has a base sequence containing n+1 bases and is complementary to the base sequence formed when multiple repeat units are extended in a 5'to 3' direction;

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L14: Entry 3 of 4 File: USPT Jul 31, 2001

DOCUMENT-IDENTIFIER: US 6268146 B1
TITLE: Analytical methods and materials for nucleic acid detection

Brief Summary Text (9):
Polymerase chain reaction (PCR) and Southern blot-based hybridization methods for the detection of predetermined nucleic acid rely upon the use of hybridizing labeled primers or probes. Such probes have been labeled and detected using radioactivity; fluorescent spectroscopic methods using fluorescent dyes, acridinium esters and digoxygenin; and absorbance spectroscopic (often visible) methods using horseradish peroxidase, jack bean urease and alkaline phosphatase. PCR products made with unlabeled primers may be detected in other ways, such as electrophoretic gel separation followed by dye-based visualization.

Brief Summary Text (13):
Some technologies for real time detection of PCR products are based on detecting nucleic acid hybrids using fluorescence resonance energy transfer (FRET; mentioned above). These technologies either indirectly measure the amplification reaction through the use of a separate, labeled probe that hybridizes with but is not incorporated into the amplification product (U.S. Pat. Nos. 5,348,853; 5,119,801; 5,312,728; 5,962,233; 5,945,283; 5,876,930; 5,723,591; and 5,691,146) or directly detect amplification products through the use of a label directly incorporated in the amplification primer(s) (U.S. Pat. No. 5,866,336).

Brief Summary Text (14):
One such FRET-based technology for real time PCR product detection is known generally as 5' nuclease PCR assay (TaqMan.RTM. assay). In this assay the decrease in fluorescence quenching resulting from the cleavage of dually-labeled probes that hybridize downstream of amplification primers is monitored in an amplification reaction. A polymerase extends the growing nucleic acid chain from the amplification primers, and degrades hybridized dually-labeled probes from their 5'-termini using the 5' to 3' exonuclease activity of thermostable polymerases such as Taq DNA Polymerase. C. Wittwer et al., Biotechniques, 22:130-138 (1997); P. Holland et al, Proc. Natl. Acad. Sci., USA, 88:7276-7280 (1991). Although complementary to the PCR product, the fluorescently-labeled nucleic acid hybrid-detecting probes used in this assay are distinct from the PCR primers. The probes are dually-labeled with both a molecule capable of fluorescence and a molecule capable of quenching fluorescence. When the probes are intact, and hybridize to an amplification template, intramolecular quenching of energy between the two fluorophores (dual labels) of the probe leads to low, background levels of fluorescent signal. When a fluorescent molecule is liberated from the proximity of the fluorescence quencher by the exonuclease activity of a DNA polymerase (e.g. Taq DNA Polymerase) during amplification, the quenching is greatly reduced leading to increased fluorescent signal. This probe is degraded by the 5'-exonuclease activity of DNA polymerase when it hybridizes downstream of polymerase on the segment of DNA template being amplified.

Brief Summary Text (30):
In one embodiment, the analytical output is obtained by fluorescence spectroscopy. Use of a wide variety of fluorescence detection methods is contemplated. In one exemplary contemplated method, an identifier nucleotide includes a fluorescent label. An identifier nucleotide is fluorescently labeled prior to, or after, release of the identifier nucleotide. It is also contemplated that other than a released nucleotide contains a label such as a fluorescent tag. When a label is on other than a released nucleotide, the release of nucleotide is ascertained by monitoring the change in length of the labeled remainder of the depolymerized probe, or by a change in fluorescence when the identifier nucleotide includes a molecule capable of quenching or enhancing fluorescence. In such an embodiment, the release of nucleotides in a

process of the invention is ascertained by a determination of a difference in the length of the polynucleotide probe, for example, by capillary electrophoresis imaged by a fluorescent tag at the 5' terminus of the probe or in a region other than the 3'-terminal region.

Brief Summary Text (31):
In an alternative embodiment the analytical output is obtained by mass spectrometry. It is preferred here that an identifier nucleotide be a nucleotide analog or a labeled nucleotide and have a molecular mass that is different from the mass of a usual form of that nucleotide, although a difference in mass is not required. It is also noted that with a fluorescently labeled identifier nucleotide, the analytical output is alternatively obtained by mass spectrometry. It is also contemplated that the analysis of released nucleotide be conducted by ascertaining the difference in mass of the probe after a depolymerization step of a process of the invention.

Brief Summary Text (32):
In another alternative embodiment, the analytical output is obtained by absorbance spectroscopy. Such analysis monitors the absorbance of light in the ultraviolet and visible regions of the spectrum to determine the presence of absorbing species. In one aspect of such a process, released nucleotides are separated from hybridized nucleic acid and other polynucleotides by chromatography (e.g. HPLC or GC) or electrophoresis (e.g. PAGE or capillary electrophoresis). Either the released identifier nucleotide or the remainder of the probe is analyzed to ascertain the release of the identifier nucleotide in a process of the invention. In another aspect of such a process a label may be incorporated in the analyzed nucleic acid.

Brief Summary Text (34):
A contemplated kit optionally further comprises a nucleic acid label. In some embodiments of a kit or composition, the identifier nucleotide comprises a fluorescent label and the probe optionally further comprises a fluorescence quencher or enhancer molecule. In other embodiments of a kit or composition, the identifier nucleotide comprises a non-natural nucleotide analog.

Brief Summary Text (64):
The term "identifier nucleotide", as used herein, refers to a nucleotide whose presence is to be detected in a process of the invention to identify that a depolymerization reaction has occurred. The particular application of a method of the invention affects which residues are considered an identifier nucleotide. For a method using ATP detection (e.g. luciferase/luciferin or NADH) wherein, during analysis, all nucleotides released in the depolymerization are "converted" to ATP with an enzyme such as NDPK in the presence of ADP and use of the released nucleotide as the phosphate donor, all nucleotides released are identifier nucleotides. Similarly, for a method using absorbance detection that does not distinguish between nucleotides, all released nucleotides are identifier nucleotides. For a mass spectrometric detection wherein all the released nucleotides are analyzed, all released nucleotides can be identifier nucleotides; alternatively a particular nucleotide (e.g. a nucleotide analog having a distinctive mass) can be detected. For fluorescence detection, a fluorescently-labeled nucleotide is an identifier nucleotide. The nucleotide can be labeled prior to or after release from the nucleic acid. In some cases, the release of identifier nucleotide is deduced by analyzing the remainder of the probe after a depolymerization step of the invention. Such analysis is generally by a determination of the size or mass of the remaining probe and can be by any of the described analytical methods (e.g. a fluorescent tag on the 5'-terminus of the probe to monitor its molecular weight following capillary electrophoresis).

Brief Summary Text (86):
In one contemplated embodiment of the invention, the enzyme whose activity is to depolymerize hybridized nucleic acid to release nucleotides from the probe 3'-terminal end is a template-dependent polymerase. In such an embodiment, the reverse of a polymerase reaction (pryophosphorolysis) is used to depolymerize a nucleic acid probe, and the identifier nucleotide is released most efficiently when the 3'-terminal nucleotide of the nucleic acid probe hybridizes with total complementarity to its nucleic acid target sequence. Thus, in an embodiment using the reverse of a polymerase reaction to depolymerize nucleic acid hybrid, a signal confirms the presence of a nucleic acid target sequence that has a sequence complementary to the nucleic acid

probe in order to be detected by the process of the invention. In an embodiment using fluorescent labels along with a fluorescence enhancer or fluorescence quencher the depolymerization may be observed by a change in the fluorescence emission as discussed below.

Brief Summary Text (96):
A contemplated method is particularly useful in a multiplex assay environment in which a plurality of probes is utilized to determine whether one or more of a plurality of predetermined nucleic acid target sequences is present or absent in a sample.

Brief Summary Text (99):
In a multiplex embodiment of the above process, the sample is admixed with a plurality of different nucleic acid probes, preferably after amplification of the multiple nucleic acid targets as needed. Multi-well plates common in the art presently have volumes of 100 .mu.L for 96 well plates, and 10 .mu.L for a 384 well plates. The 100 .mu.L volume is preferred for most embodiments of the present invention. In an embodiment of the invention lacking unique identifier nucleotides, the analytical output for a certain result with one of the probes is distinguishable from the analytical output from the opposite result with all of the probes.

Brief Summary Text (101):
In a contemplated multiplex embodiment, information about the presence or absence of a plurality of nucleic acid target sequences is determined using a process of the invention on a single nucleic acid sample, by admixing the sample with a plurality of nucleic acid probes for the various nucleic acid targets.

Brief Summary Text (105):
The decision of whether to analyze for released identifier nucleotide or the remaining probe is based upon the embodiment of the invention selected, and where a label is placed. In several embodiments, a conveniently detectable label is placed on the nucleic acid probe, preferably in the 3'-terminal region where the labeled nucleotide is depolymerized as a released identifier nucleotide, but alternatively in a region other than the 3'-terminal region, in such a manner that the labeled nucleotide remains on the probe after depolymerization.

Brief Summary Text (108):
Exemplary detection systems for detecting either released identifier nucleotide or the remaining probe include mass spectrometry, fluorescence spectroscopy and absorbance spectroscopy. These detection methods are equally useful for the detection of the released identifier nucleotide as they are for the remaining probe, depending on where the label is (if a label is used) or on size differential. For example, in cases where the size of the remaining nucleic acid probe after depolymerization is not easily distinguishable from the size before depolymerization, one may choose to focus on the determination of the released nucleotides. The various detection systems are discussed hereinbelow.

Brief Summary Text (111):
Nucleic acid analogs chosen for use in this aspect of the invention should not interfere with either the hybridization of the nucleic acid probe or depolymerization of the hybridized probe. Various nucleotide analogs and nucleic acid labels such as pendant fluorescent groups are well known in the art, and are known to not interfere with hybridization or polymerization. Such nucleotides and labels are commercially available and are useful in practicing the invention.

Brief Summary Text (113):
As mentioned briefly above, a mass spectrometric analysis of the nucleic acid polymers in the treated sample can be used either to identify released nucleotides or to determine whether a nucleic acid probe or target was depolymerized by ascertaining the size of the remaining nucleic acid polymer. Using mass spectrometry, the size of the probes after treatment of hybridized samples with a depolymerizing enzyme can be determined to discover whether a given probe has been depolymerized. If unique probe sizes are used in a multiplex embodiment, it is possible to distinguish which probes were depolymerized. No labels are necessary for the determination of size differences based on depolymerization, however the use of labels comprising nucleotide analogs is contemplated and can be helpful in distinguishing multiple probes. Such labels are

preferably at other than the 3'-terminal region in this embodiment. It is contemplated that a label on the probe also encompasses a non-complementary nucleic acid sequence at the 5'-terminus of the region of the probe that is complementary to the nucleic acid target. Alternatively, a label on the 5'-terminus of a probe is another molecule altogether, that isn't even a nucleotide, but merely that acts as a weight marker.

Brief Summary Text (114):
In a multiplex embodiment of the present invention, mass spectrometry can be used to resolve the presence of one or more identifier nucleotides or probe sizes in such a manner as to distinguish which probe:target nucleic acid nucleic acid hybrids were depolymerized. Preferably, this is done using multiple different identifier nucleotides in the various nucleic acid probes. Different identifier nucleotides have distinguishably different labels, or they have 3'-terminal regions with identifiably different nucleotides (e.g. only the wild type probe has G residues in the 3'-terminal region). Using such a technique, the presence of the different released identifier nucleotides is direct evidence of the presence of the nucleic acid target sequences.

Brief Summary Text (119):
In some contemplated embodiments with fluorescence spectroscopic analysis, the identifier nucleotide includes a fluorescent label. In one embodiment when the nucleotide has a fluorescent label, the analytical output is obtained by fluorescence spectroscopy. In an alternative embodiment when the nucleotide has a fluorescent label, the analytical output is obtained by mass spectrometry discussed before, because the presence of an added fluorophore alters the molecular weight of the identifier nucleotide.

Brief Summary Text (120):
In a preferred embodiment of the invention, the fluorescent label is part of a fluorescently-labeled analog of a nucleotide and that nucleotide is incorporated into the probe, preferably in the 3' terminal region to be released as the identifier nucleotide. Alternatively, the fluorescently-labeled nucleotide analog is incorporated into the probe in other than the 3'-terminal region to be analyzed as the part of the probe left after treatment with a depolymerizing enzyme.

Brief Summary Text (121):
In an alternative embodiment, a nucleic acid probe comprises a fluorescent label, either in the 3'-terminal region or not in the 3'-terminal region, and the probe further comprises a second fluorophore, capable of acting either as a fluorescence quencher or a fluorescence enhancer. Release of either the fluorescent label itself, or the fluroscence quencher/enhancer results in a change in fluorescence of the sample to generate an analytical output signaling the presence or absence of the nucleic acid target. This embodiment is discussed in more detail below.

Brief Summary Text (122):
Fluorescent nucleotide analogs are widely known and commercially available from several sources. An exemplary source is NEN.TM. Life Science Products (Boston, Mass.), which offers dideoxy-, deoxy-, and ribonucleotide analogs a labeled with fluorescein, coumarin, tetramethylrhodamine, naphthofluorescein, pyrene, Texas Red.RTM., and Lissamine.TM.. Other suppliers include Amersham Pharmacia Biotech (Uppsala, Sweden; Piscataway, N.J.) and MBI Fermentas, Inc. (Amherst, N.Y.).

Brief Summary Text (123):
An advantage to using fluorescent labels and fluorescence spectroscopy analysis is that there are multiple different labels available. Such different labels can be particularly useful in a multiplex embodiment of the invention. Different fluorescent labels are used in different probes, so that the detection of a particular fluorescently-labeled nucleotide analog as a released identifier nucleotide can be distinguished from others and used to deduce which nucleic acid targets are present.

Brief Summary Text (126):
It is contemplated that a depolymerization reaction product, such as a released identifier nucleotide or the probe that remains, can be labeled before or after depolymerization using cross-linking chemistry well known in the art with commercially available reagents. For example, fluorescein isothiocyanate and rhodamine B isothiocyanate are both available from Aldrich Chemical Company (Milwaukee, Wis.).

References to use of fluorescein isothiocyanate in labeling biological molecules include Nature, 193:167 (1962); Meth. Enzymol., 26:28 (1972); Anal. Biochem., 57:227 (1974); Proc. Natl. Acad. Sci., USA, 72:459 (1975).

Brief Summary Text (132):
A fluorescence spectroscopic embodiment of the invention takes advantage of fluorescent resonance energy transfer (FRET). A nucleic acid probe has two fluorophore labels (e.g. A and B) in proximity to one another in the intact probe such that exciting A with a laser will emit light which excites B, resulting in a detectable fluorescent emission from B. Depolymerization of a probe:hybrid duplex according to the invention results in the release of a nucleotide harboring a fluorophore label, such that they are no longer in resonant proximity with one another. The release of nucleotide results in a decrease in the fluorescent emission from B upon laser excitation of A. It is contemplated that the depolmerization process is monitored either in real time (i.e. at multiple time points) or at an end-point (i.e. at a set time after the depolymerization reaction begins). In one contemplated embodiment, both fluorophores are in the 3'-terminal region of the probe. In an alternative contemplated embodiment, one fluorophore is in the 3'-terminal region and a second fluorophore is elsewhere in the probe. The disclosures of U.S. Pat. Nos. 5,348,853; 5,119,801; 5,312,728; 5,962,233; 5,945,283; 5,876,930; 5,723,591; 5,691,146; and 5,866,336 disclosing fluorophore labeled oligonucleotides are incorporated herein by reference.

Brief Summary Text (133):
In a related fluorescence enhancement spectroscopic embodiment, there are two probes that are distinguishable. One probe will be depolymerized on a wild type target, and the other probe will be depolymerized on a mutant target. It is contemplated that the reactions with the two probes can be conducted in a single reaction vessel. In another related multiplex fluorescent enhancement spectroscopic environment, there are multiple distinguishable probes to different nucleic acid targets. Depolymerization results in a decrease in fluorescence.

Brief Summary Text (134):
In an alternative fluorescence quenching spectroscopic embodiment, there are two fluorophores on a probe, one that quenches the fluorescence of the other when they are in proximity with one another. Such interacting fluorescent molecules, including labeled nucleotide analogs are known in the art [Lee et al., Nucleic Acids Res., 21:3761-3766 (1993); Bassler et al., App. Environ. Microbiol., 61:3724-3728 (1995); Livak et al., PCR Methods Applic., 4:357-362 (1995); and Livak et al., Nature Genet., 9:341-342 (1995)] and commercially available, for example 6-carboxy-fluorescein (6-FAM; fluorescence emission observed at 518 nm) and 6-carboxytetramethylrhodamine (TAMRA). Thus, when the probe is intact, the fluorescence of one fluorophore is quenched. Depolymerization of the probe results in removal of the quencher from quenching proximity, resulting in an observed increase in fluorescence. The multiplex embodiments discussed above with fluorescence enhancement are also contemplated for this embodiment with fluorescence quenching, once again with real-time or end-point fluorescence detection.

Brief Summary Text (140):
In an illustrative embodiment, a multiplexed assay for the presence of several different nucleic acid target sequences in a sample is analyzed by absorbance spectroscopy. Several labeled probes to various nucleic acid target sequences are added to a nucleic acid sample. The labels on the probes may be various nucleotide analogs, a different one for each probe. A depolymerizing enzyme is added, such as Klenow exo-, releasing the labeled nucleotides and other nucleotides from the 3'-termini of probes hybridized to target sequences when the 3'terminal nucleotide is matched.

Brief Summary Text (141):
The reaction solution is loaded onto a pre-equilibrated High Pressure Liquid Chromatography (HPLC) column and eluted under conditions that separate the nucleotide analogs from the natural nucleotides. Useful media for chromatographic separation of nucleotides, bases, and nucleosides include reverse phase media, such as a reverse phase C18 column or ODS-80T.sub.M or ODS-120T TSK-GEL by TosoHaas (Montgomeryville, Pa.), anion exchange media, such as DEAE-25SW or SP-25W TSK-GEL by TosoHaas

(Montgomeryville, Pa.), or affinity media, such as Boronate-5PW TSK-GEL by TosoHaas (Montgomeryville, Pa.). Example 1 illustrates an embodiment of the present invention using HPLC.

Brief Summary Text (149):
In some embodiments, a contemplated kit optionally further comprises at least one nucleic acid probe, said nucleic acid probe being complementary to a nucleic acid target sequence and comprising an identifier nucleotide. In some embodiments, the nucleic acid probe includes at least one label, as discussed above. In some embodiments, the nucleic acid probe comprises a nucleotide analogue.

Brief Summary Text (155):
The invention contemplates kits optionally containing a nucleic acid probe for a nucleic acid target of interest, said nucleic acid probe being complementary to a predetermined nucleic acid target and comprising an identifier nucleotide. In another embodiment, the kits contain multiple probes, each of which contain a different base at an interrogation position or which are designed to interrogate different target DNA sequences. In a contemplated embodiment, multiple probes are provided for a set of nucleic acid target sequences that give rise to analytical results that are distinguishable for the various probes. Exemplary probes and groups of probes are provided in the parent application, listed above and incorporated herein by reference.

Brief Summary Text (156):
It is contemplated that a kit contain a vessel containing a purified and isolated enzyme whose activity is to release one or more nucleotides from the 3' terminus of a hybridized nucleic acid probe, and a vessel containing pyrophosphate. In one embodiment, these items are combined in a single vessel. It is contemplated that the enzyme is either in solution or provided as a solid (e.g. as a lyophilized powder), the same is true for the pyrophosphate. Preferably, the enzyme is provided in solution. It is further contemplated that a kit contain a vessel containing one or more nucleic acid probes, said nucleic acid probe being complementary to a predetermined nucleic acid target sequence and comprising an identifier nucleotide. Some contemplated kits contain labeled nucleic acid probes. Other contemplated kits further comprise vessels containing labels and vessels containing reagents for attaching the labels.

Brief Summary Text (158):
As discussed above, the nucleic acid probe optionally comprises a label, or a nucleotide analog. The enzymes and probes are as discussed above with respect to the kits. Thus, in some embodiments of a kit or composition, the identifier nucleotide comprises a fluorescent label and the probe optionally further comprises a fluorescence quencher or enhancer. As mentioned above, exemplary useful fluorophores are Fluorescein, 5-carboxyfluorescein (FAM), 2'7' dimethoxy-4'5'-dichloro-6-carbosyfluorescein (JOI), rhodamine, 6-carboxyrhodamine (R6G), N,N,N,N-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), and 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS). In other embodiments of a kit or composition, the identifier nucleotide comprises a non-natural nucleotide analog.

Detailed Description Text (46):
The interrogation probe is designed to have a fluorescent label attached to the 3'-terminal nucleotide in a manner such that the label does not interfere with the ability of the depolymerizing enzyme to remove the nucleotide from the probe. Such fluorescent tags, such as fluorescein or rhodamine, are incorporated into the probe during synthesis with the fluorescent molecule attached to the phosphoramadite nucleotide with a linker of at least 6 carbons (Glen Research). Additionally, in this example an identical, but unlabeled, probe is used and released nucleotides are fluorescently labeled only after the nucleotide is released from the probe by a process of the invention.

Detailed Description Text (54):
Fifty microliters of master mix are then added. The composition of the master mix containing Klenow exo- is described in Example 3 with the exception that there is no

ADP and no NDPK. The reaction then proceeds at 37.degree. C. for 15 minutes. The two reactions that do not contain fluorescent-labeled nucleotides are further treated to label the released nucleotides with a fluorescein tag. Jain, R. et al., Biochem. Biophys. Res. Comm., 200:1239-1244 (1994); Shuker, D. et al., IARC Sci Publ., 124:227-232, (1993).

Detailed Description Text (59):
Multiplex Interrogation Using Fluorescent Labels

Detailed Description Text (60):
This example demonstrates that nucleotides released from the 3'-terminus of multiple probes, each hybridized to a target nucleic acid of interest, by a process of the invention can be detected by mass spectrometry or by fluorimetric HPLC and thereby provide evidence of the presence or absence of the target nucleic acid in a nucleic acid sample or of a specific base at an interrogation position of the target.

Detailed Description Text (61):
Each interrogation probe is designed to have a different fluorescent label attached to the 3'-terminal nucleotide in a manner such that the label does not interfere with the ability of the depolymerizing enzyme to remove the nucleotide from the probe. Fluorescent tags, such as fluorescein or rhodamine, are incorporated into the probe during synthesis with the fluorescent molecule attached to the phosphoramadite nucleotide with a linker of at least 6 carbons (Glen Research).

Detailed Description Text (75):
The interrogation probe is designed to have a fluorescent label attached to the 5'-terminal nucleotide. Fluorescent tags, such as fluorescein or rhodamine, are incorporated into the probe during synthesis with the fluorescent molecule attached to the phosphoramadite nucleotide present at the 5'-end of the oligonucleotide that is used as a probe (Glen Research).

Detailed Description Text (77):
The prothrombin interrogation probes are 11265 (SEQ ID NO:6), that is totally complementary to a segment of the mutant prothrombin sequence, and 11266 (SEQ ID NO:7), that is totally complementary to a segment of the wild-type prothrombin sequence. Each of these probes has a destabilizing mutation eight bases from the 3' end. And each of these probes has a label at its 5'-end, incorporated during synthesis of the probe as described above.

Detailed Description Text (82):
The solutions are then split in half and analyzed using two different methods. In one method, the size of the labeled probe in the solutions is analyzed by silicon desorption ionization mass spectroscopy (Wei, J. et al. Nature. 399:243-246, 1999). This method is sensitive to femtomole and attomole levels of analyte. The samples are prepared for spectrometry as described in that paper. Essentially, analytes are dissolved in a deionized water/methanol mixture (1:1) at concentrations typically ranging from 0.001 to 10.0 .mu.M. Aliquots (at least 0.5 to 1.0 .mu.L, corresponding to at least 0.5 femtomol to 100 picomol analyte) of solution are deposited onto the porous surfaces and allowed to dry before mass spectrometry analysis.

Detailed Description Text (84):
In a second method, the size of the denatured labeled probe strand in the solution is analyzed by HPLC using a fluorescence detector as described in Jain, et al. Biochem. Biophys. Res. Commun. 200:1239-1244 (1994) or Levitt, B. et al. Anal. Biochem. 137:93-100 (1984). The size of the denatured labeled probe strand is confirmed on an ABI 377.

Detailed Description Text (85):
The size of the labeled probe strand present in the denatured solution indicates whether or not a nucleotide was released from the 3'-terminus of the probe, and therefore whether a match or mismatch base pair existed at the 3' terminus of the probe/template hybrid. For the denatured solution containing wild-type probe, the observance of a labeled probe that is shorter than the length of the original probe indicates that there is a matched base at the 3'-terminus of at least one allele in the original sample and therefore, that at least one allele in the original sample is

wild-type. For the denatured solution containing mutant probe, the observance of a labeled probe that is shorter than the length of the original probe indicates that there is a matched base at the 3'-terminus of at least one allele in the original sample and therefore, that at least one allele in the original sample is wild-type. In both cases, the analytical output is quantified to determine whether the genotype is homozygous or heterozygous at that locus.

Detailed Description Paragraph Table (9):
40 .mu.L each of the two PCR products 1.5 nmol each of the wild type or each of the mutant labeled interrogation oligos

Other Reference Publication (42):
Axton, et al., "A Single-Tube Multiplex System for the Simultaneous Detection of 10Common Cystic Fibrosis Mutations", Human Mutation, 5:260-262 (1995).

CLAIMS:

3. The method according to claim 2 wherein said released identifier nucleotide comprises a fluorescent label.

11. The method according to claim 10 wherein said released identifier nucleotide includes a fluorescent label.

19. The method according to claim 18 wherein said released identifier nucleotide includes a fluorescent label.

31. The method according to claim 29 wherein said identifier nucleotide includes a fluorescent label.

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☑ 3. 6268146 . 22 Nov 99; 31 Jul 01. Analytical methods and materials for nucleic acid detection. Shultz; John William, et al. 435/6; 435/91.2 435/91.5 436/173 436/501 536/24.31 536/24.32. C12Q001/68 C12P019/34 G01N024/00 C07H019/04.

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